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[54] Title of Invention: Carcinostatic Agent

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SPECIFICATIONS

1. Title of the Invention

Carcinostatic Agent

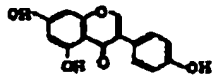
2. Claims

A carcinostatic agent whose active principle is 5,7,4'-trihydroxyisoflavone (genistein)

3. Detailed Description of the Invention

Applicable field of industry

This invention relates to a carcinostatic agent whose active principle is 5,7,4'-trihydroxyisoflavone (commonly known as genistein), and which is represented by the formula



Prior art

Genistein is a known compound, recorded in 1951 on page 3447 of the *Journal of the Chemical Society*. According to this article, genistein is a compound separated from a type of clover (*Trifolium subterraneum L.*), and is reported to have a weak oestrogen effect. However, absolutely no carcinostatic action is reported.

Action and effects of the present invention

The inventors of the present invention recognized a substance in the fermentation products of microorganisms belonging to the *Pseudomonas* genus isolated from soil as having

carcinostatic action and, as a result of further research into the substance, determined that the substance was genistein, and perfected the present invention.

The following describes the carcinostatic properties and toxicity of the compound of the present invention.

← Tumour cell propagation inhibiting action and DNA synthesis inhibiting action

The carcinostatic properties of genistein were investigated through the following experiments on the inhibition of the propagation and inhibition of the synthesis of DNA in the following experimental tumour cells.

(a) Tests on inhibiting propagation of rat cells transformed with Rous' sarcoma virus (RSV-3Y1)
(b) Tests on inhibiting propagation of human epidermal carcinoma cells (A431 cells)
(c) Tests on inhibiting propagation of rat cells transformed with SV40 virus (SV 40-3Y1 cells)
(d) Tests on inhibiting synthesis of DNA in mouse mast cell carcinomas (P815 cells)
(e) Tests on inhibiting synthesis of DNA in mouse thymuses (EL-4 cells)

Test methods and results of tests

The tests (a), (b) and (c) above were performed in the following manner.

The RSV-3Y1 cells (a), A341 cells (b) and SV40-3Y1 cells (c) were cultured in *Dulbecco MEM* (manufactured by Nippon Suisan KK) containing 2% calf embryo serum (manufactured by Gibco) and varying concentrations of genistein. Four levels of genistein concentration were employed: none added, 1 μg/ml, 3 μg/ml and 10 μg/ml. The numbers of live cells per dish were counted by means of Trypan Blue after the first, second, third and fourth days. The results are set out in Figure 1 (a) to (b).

As can be seen from Figure 1, genistein was found to have the effect of arresting cell propagation at levels of addition of from 1 μg/ml to 3 μg/ml, and the effect of inhibiting cell propagation was very strong at a level of addition of 10 μg/ml.

The methods of tests (d) and (e) above were as follows.

P815 cells (d) and EL-4 cells (e) were suspended in RPMI 1640 culture (manufactured by Nippon Suisan KK) to which 2% 56° C 30 minute inactivated calf embryo serum (manufactured by Flow Laboratories) and 80 μg/ml gentamycin (Manufactured by Essex (Japan) KK) had been added, and a final cell count of 2×10^5 cells/ml was produced. 200 μl per well of the cell suspension solution was placed in a 96 well flat-based microplate (manufactured by Sumitomo Bakelite), and genistein was added in the following concentrations: none, 1 μg/ml, 3 mg/ml and 10 μg/ml. The plate was cultured for 24 hours in a 37° C 5% CO_2 culture system, whereupon 0.1 μCi/well of [^{3}H] Thymidine (manufactured by Amersham (Japan) KK) was added, and the solutions were cultured for a further 18 hours. The cells in each well were collected by glass fibre filter (Whatman GF/C) , and the filters were dried and placed in scintillation vials, toluene scintillator was added, and the uptake by [^{3}H] Thymidine was measured by means of liquid scintillation counter. The results are set out in Figure 2.

As can be seen from Figure 2, when 3 μg/ml of genistein was present in the culture solution, the uptake of Thymidine by the P815 cells was restricted to approximately 50%, while at a level of 10 μg/ml, the uptake of Thymidine by the P815 and also EL-4 cells was completely inhibited.

↑ Inhibiting action against tyrosine-specific phosphorylase

The inhibiting action of genistein against various enzymes was measured in relation to the following three types of tyrosine-specific protein kinase (a to c), two types of serine and threonine protein kinase (d and e) and other enzymes (f to h).

(a) Tyrosine-specific phosphorylase derived from mouse carcinoma virus (Src gene $pp60^{src}$)

(b) Human epidermal carcinoma cell propagation factor receptor (EGF receptor, A431 cell) tyrosine-specific phosphorylase

(c) Tyrosine-specific phosphorylase derived from cat carcinoma virus (fes gene, $pp110^{fes}$)

(d) c-AMP dependent protein kinase

(e) Phosphorylase kinase

(f) Phosphodiesterase

(g) Na^+, K^+-ATPase

(h) 5'-nucleotidase

Of these, (a) to (c) related to tyrosine-specific phosphorylase derived from carcinoma genes, and (d) and (e) to serine and threonine protein kinase.

The method of measurement of the inhibiting action of genistein on the enzyme activity of these, and the results of such measurements, are shown below.

Method of measurement

(a) Method of measurement of activity of tyrosine-specific phosphorylase derived from Rous' sarcoma virus (Src gene $pp60^{src}$) (see M.S. Collet and R.L. Elligson: *Proceedings of the National Academy of Sciences of the USA*, vol.75 pp2021-2024 (1978))

3Y1 cells (fibroblasts derived from rat embryo kidney) transformed by Rous' sarcoma virus (RSV) are grown, and after washing with RIPA buffer (0.5% NP40, 0.1% sodium deoxycholate, 50 mM Tris-HCl pH 7.2, 1 mM phenylmethyl sulphonyl fluoride (PMSF), 0.15M NaCl) is added, and the cells are solubilized by being allowed to stand

for 30 minutes at 0° C. This is centrifuged for 20 minutes at 100,000 x g and is then inoculated with RSV, antiserum obtained from carcinoma-infected rabbits is added and the mixture is incubated for from 30 minutes to 1 hour at 0° C and the $pp60^{src}$ and antibodies are reacted together. The immune complex is concentrated by mixing with protein A-Sepharose-4B (manufactured by Pharmacia) and is then washed in RIPA buffer. The $pp60^{src}$-antibody- protein A-Sepharose-4B complex so formed reacts for 5 minutes at 30° C in 20 mM Pipes-NaOH pH 7.2, 5 mM $MgCl_2$, 1 mM DTT and 10 μm [γ-^{32}P] ATP (2 mCi/mmol), the protein kinase reaction is performed, whereupon a reaction halting solution containing SDS is added, the mixture is boiled for 3 minutes and the reaction is halted. The reaction solution is subjected to electrophoresis with 8% SDS-polyacrylamide gel, and after autoradiography, the radiation from the $pp60^{src}$ is measured by means of a liquid scintillation counter, and the phosphorylation reaction is quantified.

(b) Method of measurement of activity of tyrosine-specific phosphorylase from human epidermal carcinoma cell proliferation factor receptor (EGF receptor, A431 cell) (See S. Kornin G. Carpenter, and L. King: *Journal of Biological Chemistry*, vol. 255, pp.4834-3842 (1980))

Cell membranes prepared from human epidermal carcinoma cells (A431 cells) that are known to contain large numbers of EGF receptors are used as the enzyme source. A reaction solution containing genistein, 20 mM Pipes-NaOH pH 7.2, 10 mM $MgCl_2$, 3 mM $MnCl_2$, 1 mM DTT, 10μM (γ-^{32}P) ATP (2mCi/mmol) and A431 cell membrane (protein content 10 μg) is allowed to react together in 50 μl for 5 minutes, whereupon the reaction is halted, whereupon the reaction solution is subjected to electrophoresis with 8% SDS-polyacrylamide gel, and after analysis by autoradiography, the EGF receptors of molecular weight of 170,000 were examined for the presence of phosphorylation. The EGF receptors were further isolated, and the radiation was measured by liquid scintillation counter, and the extent of phosphorylation was measured.

- Method of preparation of cell membranes from A431 cells

A431 cells propagated in *Dulbecco* MEM (manufactured by Nippon Suisan KK) containing 7% calf embryo serum (manufactured by Gibco) were collected, and cell membrane follicles were prepared by the method of Rowen et al. (See Stanley Rowen, Hiroshi Ushiro, Krista Stosiek and Michael Cingaz: *Journal of Biological Chemistry*, vol. 257, pp.1523-1531 (1982)).

(c) Method of measurement of activity of tyrosine-specific phosphorylase from cat sarcoma virus (fes gene, $pp110^{fes}$) (See R.A. Feldman, T. Hanafusa and H. Hanafusa: *Cell*, vol. 22, pp.757-765 (1980))

Rat 3Y1 cells transformed with cat sarcoma virus and these cells were inoculated, and serum from cancer-bearing Fisher rats was used, and the protein kinase of immune precipitated $pp110^{fes}$ was measured after the same manner as for $pp60^{src}$.

(d) Method of measurement of activity of c-AMP-dependent protein kinase

c-AMP-dependent protein kinase prepared from rabbit muscle (protein content 4 µg) (manufactured by Sigma) reacts for 5 minutes at 30° C in 50 µl of a reaction solution containing 50 mM Hepes-NaOH pH 7.5, 10 mM $MgCl_2$, 4 µM [γ-^{32}P] ATP (2 mCi/mmol), 6 mg/ml histone type IIA (manufactured by Sigma), 10 µm c-AMP and genistein. This was spotted onto 2 x 2 cm Whatman filter paper P81, the filter paper was rinsed four times for 5 minutes on each occasion in 50 mM NaCl, and then was rinsed again with acetone, and the radiation was measured by means of a liquid scintillation counter.

(e) Method of measurement of phosphorylase-kinase activity

40 mM tris-HCl pH 7.4, 100 µm $CaCl_2$, 1 mM DTT, 10 mM $MgCl_2$, 10 µM [γ-^{32}P] ATP (2 mCi/mmol), 10 µg phosphorylase-b (manufactured by Sigma), rabbit muscle phosphorylase kinase (protein content 2 µg) (manufactured by Sigma) and genistein were reacted together in a 50 µl reaction solution for 5 minutes at 30° C, whereupon a reaction halt solution containing SDS was added, and the solution was boiled for 2 minutes at 100° C to halt the reaction. The phosphorylation of the phosphorylase-b was measured by 8% SDS-polyacrylamide gel electrophoresis - autoradiography of the reaction solution, followed by measurement of the separated phosphorylase-b by means of a liquid scintillation counter.

(f) Measurement of the activity of phosphodiesterase

50 mM tris-HCl pH 7.5, 8 mM $MgCl_2$, 0.8 mM EDTA, 0.02 mM DTT, 5 mM c-AMP (manufactured by Sigma), cow heart phosphodiesterase (protein content 10 µg) (manufactured by Sigma) and genistein in 50 µl in a reaction solution were reacted for 30 minutes at 37° C.

50 µl of 10% TCA was added and the reaction was halted, the solution was centrifuged for 10 minutes at 5,000 rpm and 90 µl of the supernatant so derived was measured for phosphorus. The phosphorus colour reaction was measured by 660 nm absorbance after the addition to the supernatant solution of 3 µl of 1% *Triton X-100*, 350 µl of distilled water and 50 µl of 5N aqueous sulphuric acid containing 2.5% ammonium molybdate and allowing to stand for 20 minutes.

(g) Measurement of the activity of Na^+, K^+ Tpase

Na^+, K^+ Tpase was prepared by the method of Kawamura et al. (see Kawamura, Ota and Nagano: *Journal of Biochemistry*, vol. 87, pp.1327-1333 (1980)): The outer medulla of dog kidney was ground in a buffer containing 50 mM imidazole pH 7.4, 0.25 M sucrose, 1 mM EDTA, and 0.1 mM ATP by polytron (manufactured by Kinematica) and then was ultracentrifuged to provide a microsome fraction that was extracted by means of SDS.

(h) Measurement of the activity of 5'-nucleotidase

A reaction solution containing 55 mM tris-HCl pH 8.5, 5.5 mM $MgCl_2$, 1.1 mM ATP, 10 mM potassium sodium tartrate, 5'-nucleotidase (snake venom) (manufactured by Sigma) and genistein was reacted in 50 µl for 3 minutes at 37° C, and the phosphorus levels of the reaction product were measured in the same manner as for phosphodiesterase.

Results

Inhibiting action of genistein in relation to various enzymes

Enzyme	ID_{50} (µg/ml)
(a) $pp60^{src}$ protein kinase	0.8
(b) EGF receptor protein kinase	0.7
(c) $pp110^{fms}$ protein kinase	6.5
(d) c-AMP dependent protein	>100

kinase	
(e) Phosphorylase kinase	>100
(f) Phosphodiesterase	>100
(g) Na^+, K^+-ATPase	>100
(h) 5'-nucleotidase	>100

ID_{50}: Level at which 50% inhibition occurs

As is clear from the above results, genistein has a specifically inhibiting action against tyrosine-specific phosphorylase derived from carcinoma genes.

Tyrosine-specific phosphorylase is believed to contribute to the propagation of carcinoma cells, and hence the recognition of a specific inhibiting effect against the action of this enzyme is the background to the carcinostatic effect of genistein.

→ C57BL/6 strain mice were injected in the abdominal cavity with genistein, and the acute toxicity of the genistein was examined. LD_{50} was found to be not less than 500 mg/kg.

In view of the above results of tests on its inhibiting effect on the propagation of tumour cells, its inhibiting effect on DNA, and its inhibiting effect on tyrosine-specific phosphorylase, genistein has an excellent carcinostatic effect, and moreover possesses a low toxicity, and thus is of value as a carcinostatic in the treatment of carcinomas in humans and animals, in the treatment of symptoms associated with metastasis of carcinomas, and in the prevention of relapse in carcinoma cases.

The clinical dosage of genistein is from 200 mg to 1000 mg of the active component per adult per day, administered in from one to four doses. The amount administered may be adjusted appropriately according to the individual circumstances of the patient, such as condition and age and so forth.

Genistein may be administered in isolation or in combination with other chemical treatment agents or immunological agents. Chemical treatment agents that may be employed in combination with genistein include cyclophosphamide, vinblastine, vincristine, adriamycin, 6-mercaptopurine, 5-fluorouracil, mytomycin C, pleomycin,

aclasinomycin, neocarzinostatin, cytosine arabinomide, actinomycin D, and nitrosourea and so forth. Immunological agents that may be employed in conjunction with genistein include for example creatine, BCG, [illegible], lentinan, interferon and interleukin and so forth. When genistein is employed in conjunction with other pharmaceuticals, the dosage of genistein is appropriately 1 of genistein to between 0.001 and 10 times the pharmaceutical employed in conjunction.

The dose of genistein may be prepared in form for oral administration (tablet, capsule or liquid) or non-oral administration (for rectal administration, inoculation or pellet). Such dose of genistein may be prepared as a combination with any commonly employed carrier or vehicle blended in the normal manner. Any generally employed carrier or vehicle may be employed, such as for example, in the case of tablets, water, fructose, lactose, gum arabic, gelatine, mannitol, starch paste, magnesium trisilicate, milk, maize starch, colloidal silica, potato starch or urea and the like. In liquid form, an aqueous or oleaginous suspension, solution, syrup, or elixir may be employed, and thee are prepared in the normal manner. For rectal administration, the genistein may be supplied as a suppository, and the base may be any normally employed base such as for example, polyethylene glycol, lanolin, cocoa fat, or *Witefzol*® (manufactured by Dynamit-Nobel) and so forth.

4. Simplified description of the drawings

(1) Figure 1 (a), (b) and (c) show the inhibiting effects of genistein on the propagation of RSV-3Y1 cells, A431 cells and SV40-3Y1 cells.

(2) Figure 2 shows the inhibiting effects of genistein on DNA synthesis in P 815 cells and EL-4 cells.

Figure 1 (a)

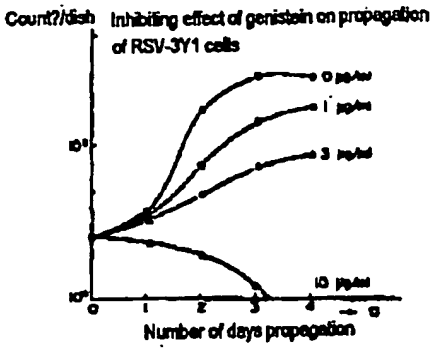


Figure 1 (b)

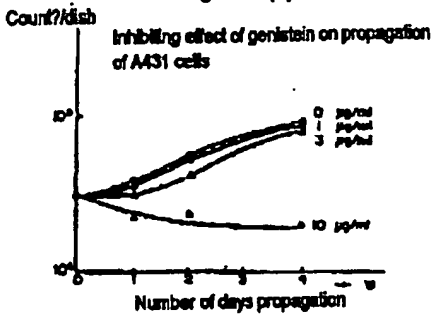
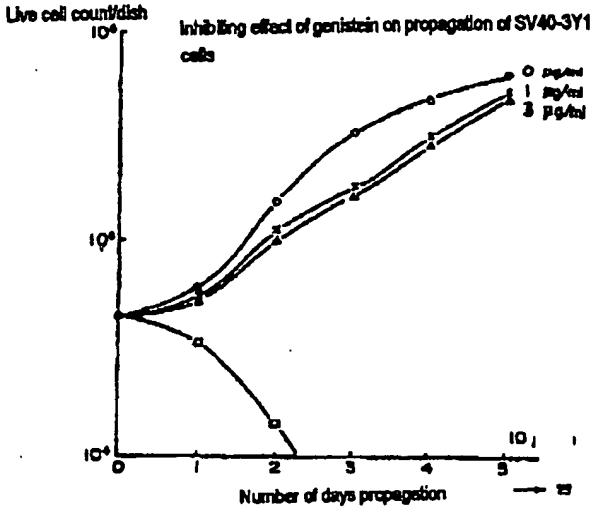


Figure 1 (c)



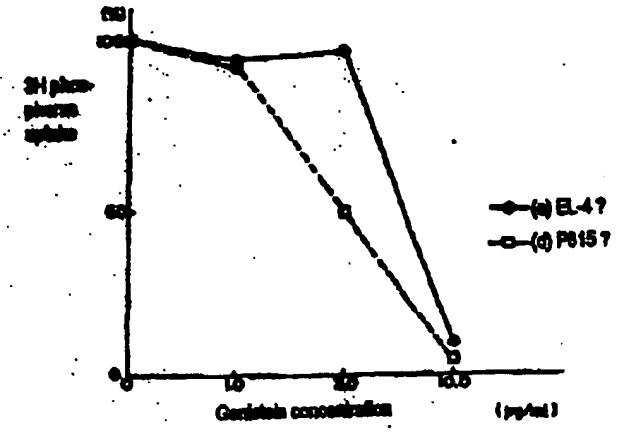


Figure 2

Amendment (Voluntary)

May 23 1985

Mr Manabu Shiga
Director, Patent Agency

1. Statement of Matter
Patent Application No. 89770 of 1985

2. Title of Invention
Carcinostatic Agent

3. Person making Amendment

Standing in matter:	Patent Applicant
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5. Subject of Amendment
Detailed Description of Invention within Specifications

6. Contents of Amendment
See attached

(1) [English] Page 2 line 35 '(a) to (b)' changed to '(a) to (c)'

(2) [English] Page 3 line 19 'Src' changed to 'src'

(3) [English] Page 3 line 39 'Src' changed to 'src'

(4) [English] Page 4 line 4-5 'reacts' changed to 'is reacted'

(5) [Not relevant in English translation]

(6) [Not relevant in English translation]

(7) [Not relevant in English translation]

(8) [Not relevant in English translation]

(9) [English] Page 5 line 4 'reacts' changed to 'is reacted'; [Not relevant in English translation]

(10) [Not relevant in English translation]

(11) [Not relevant in English translation]

(12) [Not relevant in English translation]

(13) [English] Page 6 line 5 '3 minutes' changed to '30 minutes'

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